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1. Sier et al, Gastroenterology, 1994, 107:1449-1456)

2.Ganesh et al, Cancer Res., 1994, 54:4065-4071).

3. Janicke et al, Sem. Throm. Hemostasis, 1991, 17:303-312)

4. Nekarda et al (Cancer Res., 1994, 54:2900-2907)

5. Grandahl-Hansen et al, 1993, Cancer Research 53:1513-1521)

6. JOURNAL OF NEURO-ONCOLOGY, (1994) Vol. 22, No. 2, pp. 139-151.

7. INTERNATIONAL JOURNAL OF ONCOLOGY, (MAR 1994) Vol. 4, No. 3, pp. 717-721.

8. Biol.Chem.Hoppe Seyler (376, No. 5, 259-67, 1995) 2 Fig. 67 Ref.

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SEMINARS IN THROMBOSIS AND HEMOSTASIS—VOLUME 17, NO. 3, 1991

Clinical Relevance of the Urokinase-Type and Tissue-Type Plasminogen Activators and of Their Type 1 Inhibitor in Breast Cancer

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Breast cancer is the most common malignant tumor and also the most common cause of death among all malignancies in women. The capacity of breast cancer cells for tissue invasion and early hematogenic metastasis is linked to the ability of these cells to dissolve components of the surrounding tumor stroma and to escape from their tissue environment. Experimental data show that cell proliferation, on the one hand, and the capacity for invasion and metastasis, on the other, are mutually independent properties of tumor cells.[1]

Under benign physiologic conditions, plasminogen activators cause the disintegration and remodeling of tissues, such as in wound healing, ovulation, or trophoblast invasion.[2–4] The central role of the urokinase-type plasminogen activator (u-PA) in tumor biology is becoming more and more evident. Tumor cells synthesize and secrete u-PA as an enzymatically inactive single-chain proenzyme (pro-u-PA) that is bound to specific receptors on the tumor cell surface.[5] After binding, it is converted to the enzymatically active two-chain form by traces of plasmin,[6] kallikrein,[7] or cathepsin B.[8] Receptor-bound u-PA converts plasminogen to plasmin, which subsequently binds to plasmin receptors located close to the u-PA receptors on the tumor cell surface.[9] Plasmin then degrades components of the tumor stroma (such as fibrin, fibronectin, proteoglycans, laminin) and activates procollagenase type IV, which is synthesized by the tumor cell, into collagenase type IV.[10,11] This enyzme degrades collagen type IV, which is a major part of the basement membrane.[12,13] This tumor cell surface-associated proteolysis is the prerequisite for the detachment of tumor cells from their surrounding tissue, which then might lead to tumor cell invasion and metastasis. Tumor cells may also synthesize the plasminogen activator inhibitor type 1 (PAI-1), which blocks the enzymatic activity of u-PA and tissue type plasminogen activator (t-PA). It is therefore plausible that PAI-1 is able to modify invasive properties of tumor cells and thus play an important role in the reimplantation of circulating tumor cells in distant locations after their detachment from the primary tumor.[14]

In order to evaluate the clinical and prognostic relevance of the just-mentioned tumor-linked mechanisms, u-PA, t-PA, and PAI-1 were measured by enzyme-linked immunosorbent assay (ELISA) in 115 tissue extracts of breast cancer and also of 30 benign breast specimens. In the present report we show that u-PA and PAI-1, but not t-PA, are potent and independent prognostic factors for relapse and overall survival in breast cancer patients. These findings are important, especially in lymph node-negative disease because such new factors are urgently needed for the individualization of the oncologic therapy.

MATERIALS AND METHODS

Details of the methods with regard to tissue extraction and ELISA techniques (u-PA, t-PA, PAI-1) were published elsewhere.[15–18] Results obtained were statistically analyzed and correlated with the clinical data as well as the course of the disease.[15–17] Localization of u-PA was performed by immunohistochemistry in formalin-fixed, paraffin-embedded breast cancer tissue sections.[15]

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RESULTS

Urokinase-Type Plasminogen Activator (u-PA)

u-PA was localized by immunohistochemistry (MAb 394, American Diagnostica, Greenwich, CT) in the cytoplasm and on the plasma membrane of the tumor cells (Fig. 1). Sections of formalin-fixed normal human kidney tissue, in which epithelial cells of the tubulus stain intensively, were always included to prove the validity of the method (Fig. 1A, B). The immunohistochemical score (staining intensity) estimated in breast cancer tissue sections parallels the u-PA content in the tumor tissue extracts, determined by ELISA (in the presence of 1% Triton X-100).[15] u-PA is about 12 times higher in extracts of cancer tissue than in extracts of normal breast tissue (Table 1). It is worth mentioning that a wide variation in u-PA content was seen among the individual tumors. Although established prognostic factors that indicate poor prognosis (lymph node involvement, negative hormone receptor status, vessel invasion) were associated with a higher u-PA content, a statistically significant correlation to established prognostic factors was not evident (Fig. 2). After a median follow-up of 26 months (range, 5 to 41), the clinical course of the disease revealed that patients with tumors of high u-PA content (>3.49 ng/mg protein) had a statistically significant higher relapse rate and shorter life expectancy than patients with low u-PA content (Fig. 3). This difference was already statistically significant after the short median time of observation of 12.5 months.[19] To calculate and

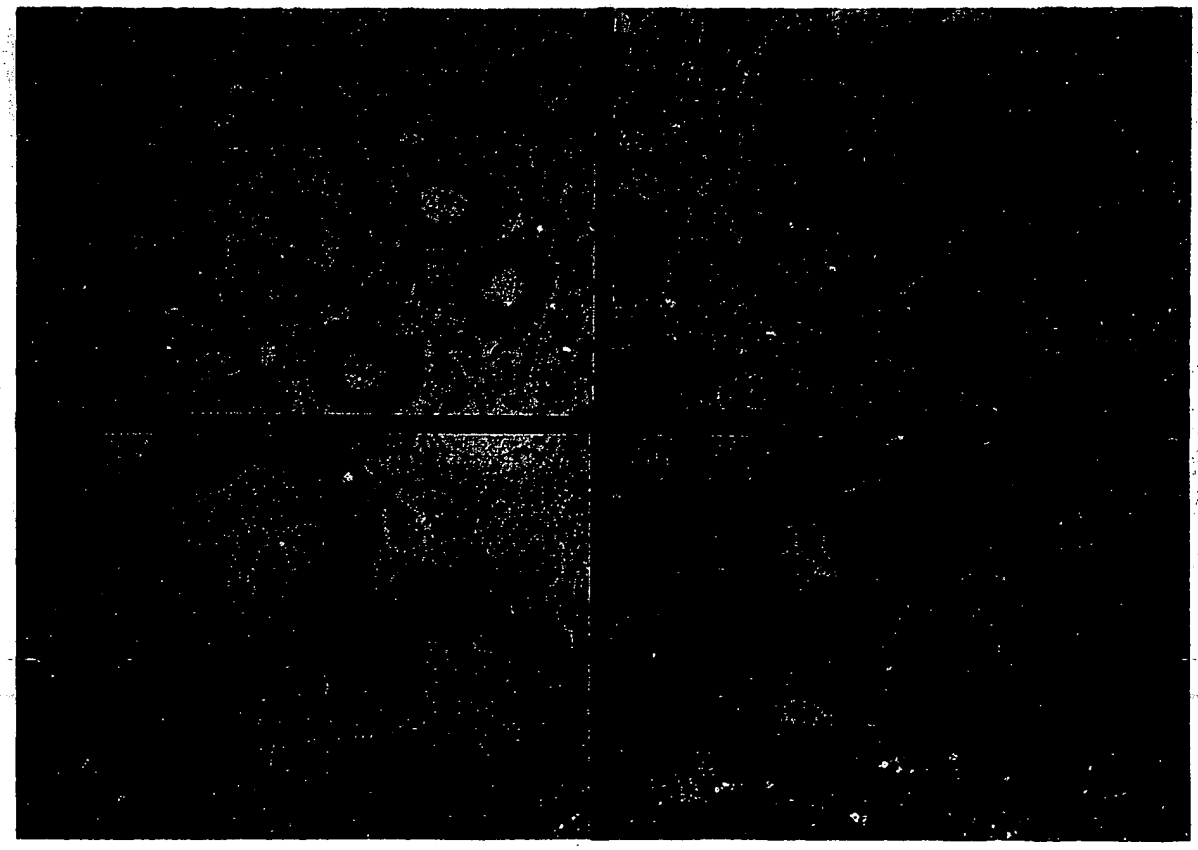

FIG. 1. Immunohistochemical staining of pro-u-PA/u-PA in breast cancer cells and in tubule cells of healthy kidney tissue by MAb 394. A,B: Formalin-fixed paraffin-embedded section of normal human kidney tissue. Immunohistochemical staining (red color) for the presence of pro-u-PA/u-PA in tubule cells. In addition to pro u-PA/u-PA (A), nuclei were stained with hematoxylin (blue color). A, × 160; B, × 1000.) C,D: Formalin-fixed paraffin-embedded section of breast cancer. Moderately differentiated invasive ductal breast carcinoma (G2). Area selected represents central part of the tumor. Immunohistochemical staining (red color) for the presence of pro-u-PA/u-PA in tumor cells (D). In addition to pro u-PA/u-PA (C), nuclei were stained with hematoxylin (blue color). C, × 400; D, × 1000.)

TABLE 1. Variables Involved in Tumor-Related Proteolysis

	Breast Cancer			*Benign Breast Tissues*			*Ratio*	*Statistical*
Antigen	*No.*	*Median (Range)*	*Mean (SD)*	*No.*	*Median (Range)*	*Mean (SD)*	*Cancer/Benign*	*Significance*
u-PA* (ng/mg)	115	2.6 (0.07-11.99)	3.2 (2.4)	30	0.22 (0-0.62)	0.24 (0.16)	12	$p < 0.001$
PAI-1 (ng/mg)	113	1.0 (0-11.33)	1.6 (1.9)	27	0.02 (0-0.52)	0.09 (0.14)	50	$p < 0.001$
t-PA (ng/mg)	115	5.3 (0-156.4)	14.6 (23.8)	30	2.97 (0-65.7)	8.57 (33.2)	—	p = not significant
Cathepsin D (pmol/mg)	107	33.4 (5.1-272)	40.1 (34.5)	28	5.80 (0.74-91)	10.67 (17.3)		$p < 0.001$

*Extraction performed with 1% Triton X-100.

compare the impact of u-PA and the other prognostic factors, multivariate analysis was performed after 12.5 and 25 months (median time of observation). u-PA, as an independent prognostic factor, had the strongest impact in predicting relapses (Table 2) and also overall survival (Table 3). The impact of u-PA was especially strong at 12.5 months. Even after 25 months, this particular role of u-PA was quite evident, although the significance of the other prognostic factors increased (Table 2). Thus, u-PA content determination in tumor tissue extracts enables the selection of high-risk and low-risk patients, even within the classic risk groups defined by locoregional tumor spread and hormone receptors. This is also true for the important group of node-negative patients (Fig. 4).

Determination of pro-u-PA/u-PA antigen content in plasma of patients with either local breast cancer or metastatic systemic disease could not be used as a tumor marker for the detection of the malignancy. Values obtained in plasma from cancer patients or healthy donors were not different (Table 4).

Plasminogen Activator Inhibitor Type 1 (PAI-1)

The PAI-1 was considerably higher in 113 extracts of breast cancer tissue compared with benign tissues (median 1.0 ng/mg versus 0.02 ng/mg protein; $p < 0.001$). A strong variation of PAI-1 antigen levels among the individual breast cancer tissues was also noted (Table 1). A statistically significant positive correlation was seen between PAI-1 and u-PA content in breast cancer ($R = 0.4$, $p < 0.001$), and of PAI-1 levels with both hormone receptor status and lymph node status: patients with positive lymph nodes had higher PAI-1 antigen levels in their primary tumors (median 1.2 ng/mg protein) than node-negative patients (median 0.8 ng/mg protein; $p < 0.05$). Patients with negative hormone receptors had statistically significantly higher levels of PAI-1 (median, 1.54 ng/mg protein) than hormone-receptor positive patients (median, 0.93 ng/mg protein; $p < 0.05$).

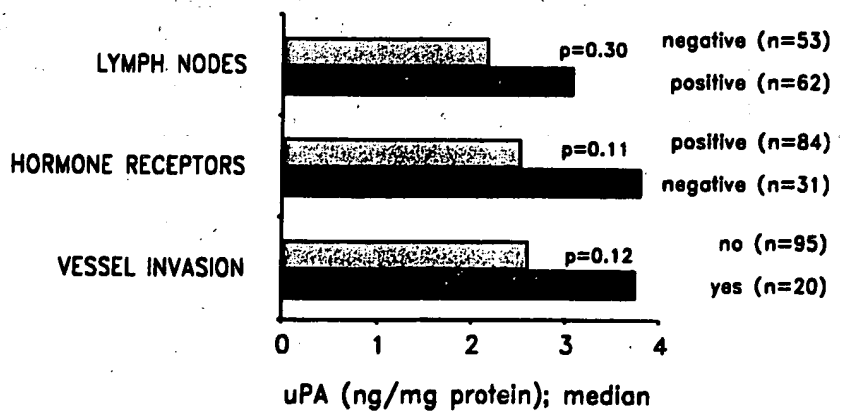

FIG. 2. Correlation of u-PA antigen determined in 115 breast cancer tissue extracts (+1% Triton X-100) to established prognostic variables. No statistically significant correlation is observed (all p values > 0.05).

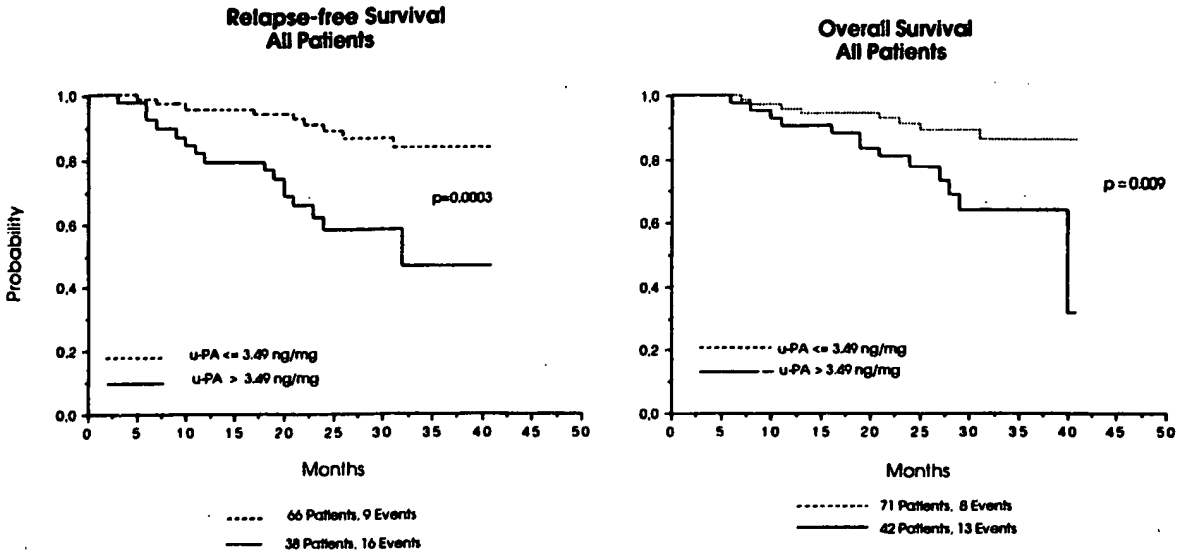

FIG. 3. Disease-free survival (left panel) and overall survival (right panel) in breast cancer patients are related to u-PA antigen content in cancer tissue extracts (ELISA). The cut-off value of u-PA (3.49 ng/mg protein) was obtained by maximizing likelihood function (Cox regression analysis). Regarding the two groups of patients with low or high u-PA, the differences in disease-free survival and in overall survival are highly statistically significant ($p = 0.0003$ and $p = 0.009$).

The clinical course of the disease was correlated with PAI-1 content, similar to u-PA. Patients with high PAI-1 antigen level in their primary tumor had a significantly higher relapse rate than patients with a low PAI-1 level (Fig. 5). Including both u-PA and PAI-1 into the multivariate analysis, both factors were determined to be independent prognostic factors for relapse and overall survival (Tables 3 to 5). Using both, u-PA and PAI-1, the group of patients at high risk for relapse could be divided even further. Patients with tumors exhibiting high u-PA (more than 3.49 ng/mg protein) and high PAI-1 content (more than 1.98 ng/mg protein) carried a maximum risk of developing relapses (Fig. 6).

Tissue-Type Plasminogen Activator t-PA

The other type of plasminogen activator, t-PA, was not significantly higher in breast cancer tissue than in normal breast tissue (Table 1). An inverse correlation between u-PA and t-PA content was found in breast cancer tissue ($r = -0.35$; $p < 0.001$) (Fig. 7). Furthermore, there was a statistically significant positive corre-

TABLE 2. Multivariate Analysis: Disease-Free Survival in 104 Breast Cancer Patients*

Variable	*Univariate p Value*	*Multivariate p Value*	*Relative Risk (95% CI†)*
u-PA (>3.49 vs <=3.49 ng/mg)	0.0004	0.0041	3.4 (1.4-7.7)
Vascular invasion (present vs absent)	0.0036	0.0332	2.8 (1.1-6.4)
Number of lymph nodes involved (>10 vs 0)	0.0151	0.0471	2.8 (1.1-7.3)
Hormone receptor status (negative vs positive)	0.0122	0.1168	2.1 (0.8-5.1)
Number of lymph nodes involved (1-10 vs 0)	0.8151	0.6130	1.3 (0.5-3.4)

*Median observation, 25 months.
†CI: confidence interval.

TABLE 3. Multivariate Analysis: Overall survival in 113 breast cancer patients*

Variable	*Univariate p Value*	*Multivariate p Value*	*Relative Risk (95% CI†)*
u-PA (>3.49 vs <=3.49 ng/mg)	0.0195	0.0016	6.0 (1.7-21.5)
PAI-1‡ (>1.98 vs <=1.98 ng/mg)	0.0103	0.0632	2.8 (0.98-8.3)
Hormone receptor status (negative vs positive)	0.0098	0.1133	—
Number of lymph nodes involved (>10 vs 0)	0.0522	0.4028	—
Number of lymph nodes involved (1-10 vs 0)	0.4691	0.899	—

*Median observation, 25 months.
†CI: confidence interval.
‡PAI-1 is correlated with lymph node and hormone receptor status.

lation between t-PA content and estrogen receptor as well as progesterone receptor (Fig. 8). Analyzing the course of the disease in regard to the t-PA content of the primary tumor, it was seen that patients with a high t-PA content tended to have a better prognosis than those with low or no detectable t-PA content. However, the difference was not statistically significant (data not shown).

DISCUSSION

The determination of proteases in tumor tissue extracts of breast cancer patients has been performed to gain more detailed information about the prognosis of the disease. The significance of determination of proteases in breast cancer tissue extracts is based on the fact that cell proliferation and the capacity of tumor cells to invade and to metastasize are two independent attributes of tumor cells.[1] The proliferative capacity of a tumor can be analyzed by determination of parameters of cell kinetics (such as ploidy, S-phase, Ki 67-antigen, thymidin labeling index). However, to gather information about the metastatic potential of tumor cells, tumor proteases have to be examined.

Our data support the notion that u-PA antigen content of the tumor tissue is an independent and strong prognostic factor for relapse and overall survival in breast cancer patients. This finding, initially published by us,[15,19] has recently been confirmed in Ireland by Duffy and coworkers[23,24] in an independent group of breast cancer patients. These scientists demonstrated as well that determination of enzymatic activity in tumor tissue extracts is of no prognostic value. Since u-PA antigen is an independent prognostic variable, determination of u-PA content enables the discrimination of subgroups of patients at high risk or low risk for relapse, even within the risk groups that were until now determined by established prognostic factors.

Tumor cells may also synthesize PAI-1, which is capable of blocking the enzymatic activity of both u-PA and t-PA. Thus, it is conceivable that PAI-1 is able to guide and even modify the invasive potential of tumor cells. As suggested by Cubellis et al,[14] due to receptor-mediated internalization of u-PA/PAI-1 complexes, the tumor cell will polarize the proteolytic activity on the cell surface. Hence, directed invasion of tumor cells into the surrounding tissue is facilitated.

Excess release of PAI-1 into tumor tissue may be of importance for the process of reimplantation of circulating tumor cells at distant loci after release from the primary site. This hypothesis is consistent with the observation that there is a lower proteolytic u-PA activity in the metastasis than in the primary tumor.[25-27] This is due to considerably higher levels of PAI-1 in the metastases, even though the u-PA antigen content in both primary tumor as well as metastasis is as high.[15] When u-PA activity is depressed by action of PAI-1, generation of metastases should be facilitated by formation of a new tumor stroma. These considerations lead one to suspect that if tumor cells of the primary tumor have a high capacity for both u-PA and PAI-1 synthesis, they may also have a high capacity to metastasize.

Indeed, this coincides with our finding that patients with high PAI-1 levels in their primary tumors had a statistically significant higher relapse rate and also a shorter overall survival than patients with low PAI-1 levels. Multivariate analysis showed that PAI-1 content enables a prognostic assessment that is independent of u-PA, although the prognostic impact of u-PA is still superior to PAI-1 (see Tables 3 and 5). Combination of

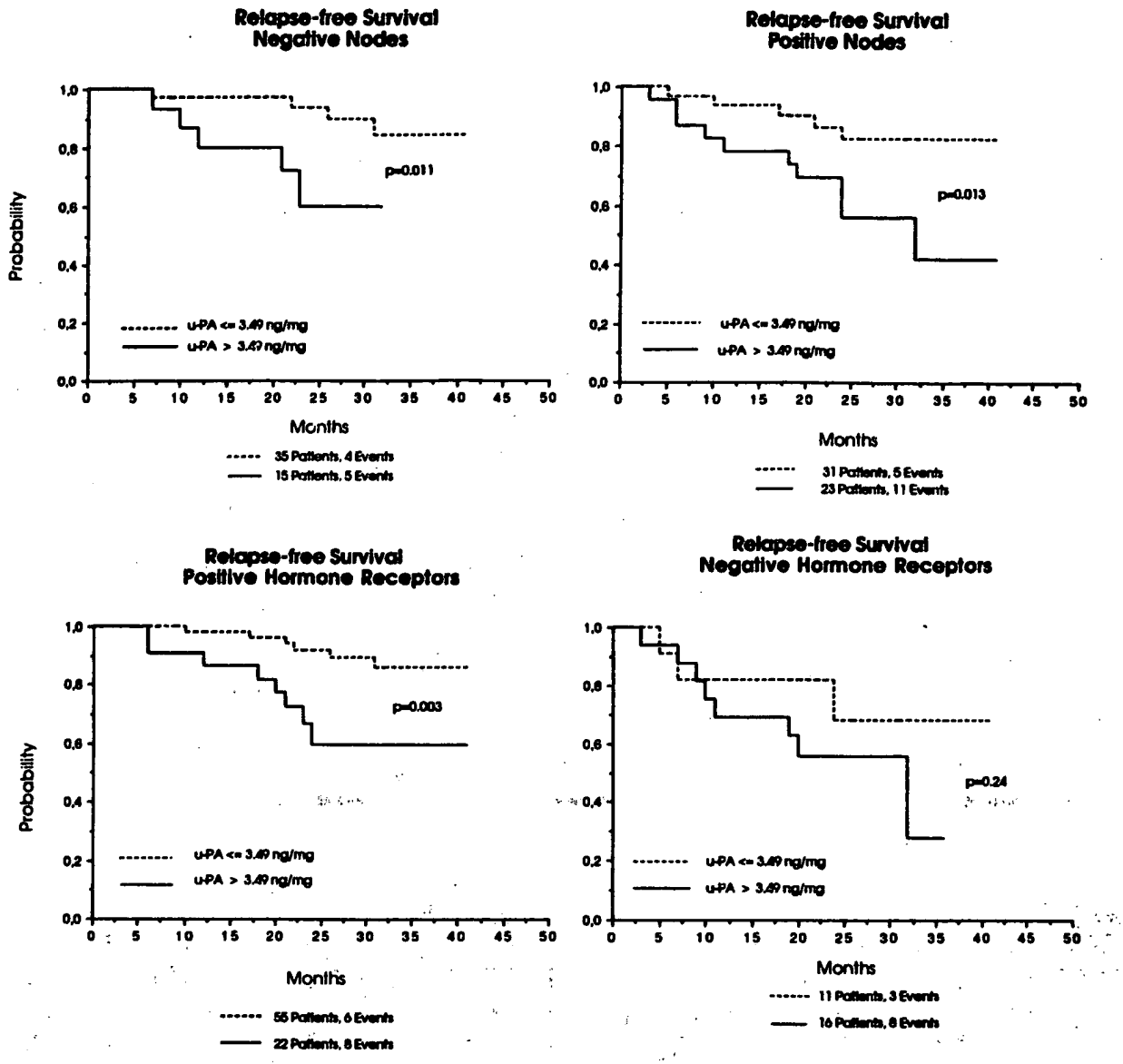

FIG. 4. Disease-free survival in breast cancer patients. Delineation of high- and low-risk patients within the prognostic groups (lymph nodes; hormone receptors) by means of u-PA.

TABLE 4. Quantitative Assessment of pro-uPA/uPA in Plasma of Patients with Breast Cancer

	pro-u-PA/u-PA Antigen (ng/ml)			
Plasma from	*No.*	*Median*	*Mean ± SD*	*Range*
Primary breast cancer*	33	0.93	0.97 ± 0.40	0.27–2.03
Metastatic breast cancer†	124	0.83	0.99 ± 0.61	0.26–3.94
Healthy donor	37	0.93	1.00 ± 0.45	0.35–2.34

*Primary breast cancer without evidence of metastases.
†Disseminated disease with various locations.

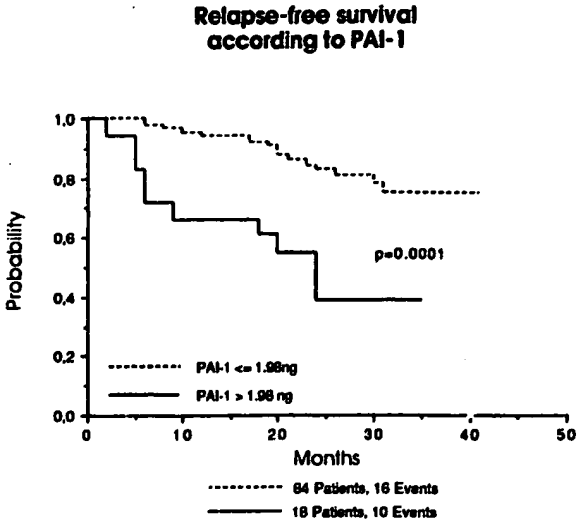

FIG. 5. Disease-free survival in breast cancer patients is related to PAI-1 antigen content in cancer tissue extracts (ELISA). The cut-off value of PAI-1 (1.98 ng/mg protein) was determined as described for u-PA. Regarding the two groups of patients with low or high PAI-1, the difference in disease-free survival is highly statistically significant (p = 0.0001).

the two strong variables u-PA and PAI-1 even further enables the delineation of patients having a low or high risk for relapse, independent of the classic risk factors. Thus, by combining u-PA and PAI-1 determination in tumor tissue extracts, a more individualized estimation of prognosis becomes possible in breast cancer patients (Fig. 6). In the future this may be of great importance for the clinical decision, whether adjuvant chemotherapy should be given or not.

In contrast to u-PA and PAI-1, t-PA was not an indicator for poor prognosis. Breast cancer patients having tumors with high t-PA content tend to have a better prognosis than those with low t-PA. This is in agreement with the results of Duffy et al,[28] who described a protective effect of high t-PA content in breast cancer. This inverse correlation between u-PA and t-PA was also confirmed for colon cancer.[29] A decrease in the differentiation of the tumor paralleled reduced t-PA values but increasing u-PA values. Similarly to the synthesis of progesterone receptors, t-PA production is stimulated by estrogens in estrogen receptor-positive breast cancer cells.[30,31] In agreement with the results of Duffy et al,[32] we found significantly higher t-PA content in estrogen receptor-positive breast cancer tissues compared with estrogen receptor-negative tumors. Additionally, we were able to demonstrate a positive correlation between the t-PA and the progesterone receptor content of the tumors, which might indicate a functional estrogen-receptor system. It remains to be demonstrated by further clinical studies whether determination of the t-PA content might enable a better prediction of the effect of hormonal therapy in breast cancer than determination of the estrogen receptor content only.

The mechanism of the t-PA effect is still not completely understood. Both plasminogen activators develop their effect by means of plasminogen activation. In contrast to t-PA, u-PA can only be effective if it is receptor-mediated. The activity of t-PA, but not that of u-PA, is stimulated by binding to fibrin.[33] There is even a higher affinity of PAI-1 to t-PA than to u-PA.[34] Interestingly, already in 1984, Markus suggested that the implantation of metastatic tumor cells could be prevented by an increased t-PA secretion, which permanently degrades the surrounding fibrin of the circulating tumor

TABLE 5. Multivariate Analysis: Disease-Free Survival in 102 Breast Cancer Patients*

Variable	*Univariate p Value*	*Multivariate p Value*	*Relative Risk (95% CI†)*
u-PA (>3.49 vs <=3.49 ng/mg)	0.0001	0.0080	4.4 (1.9-9.9)
Number of lymph nodes involved (>10 vs 0)	0.0058	0.0075	4.5 (1.7-12.9)
PAI-1‡ (>1.98 vs <=1.98 ng/mg)	0.0001	0.0346	2.6 (1.1-6.1)
Hormone receptor status (negative vs positive)	0.0016	0.0896	2.3 (0.9-5.3)
Number of lymph nodes involved (1-10 vs 0)	0.1572	—	—

*Median observation, 25 months.
†CI: confidence interval.
‡PAI-1 is correlated with lymph node and hormone receptor status.

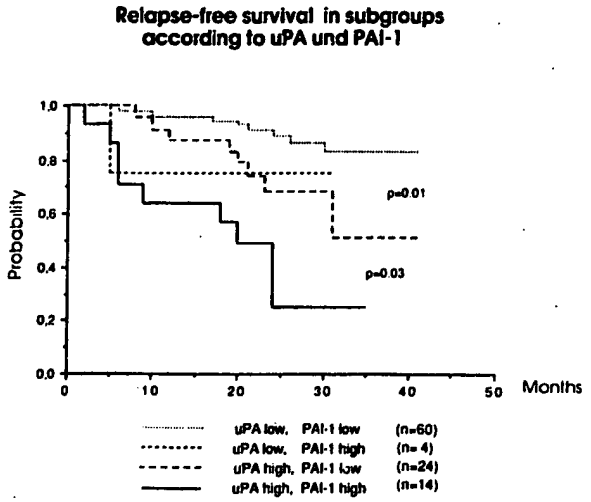

FIG. 6. Disease-free survival in patient subgroups formed by combining u-PA and PAI-1 values determined in breast cancer tissue extracts. Patients with both high u-PA (> 3.49 ng/mg) and high PAI-1 (> 1.98 ng/mg) show a maximum risk of recurrence of the disease. Even when only one of both variables is elevated, a high incidence of relapse is observed. A group of patients with a very low risk for relapse is also defined by low values of u-PA and PAI-1. The differences between the subgroups are statistically significant ($p = 0.01$ and $p = 0.03$).

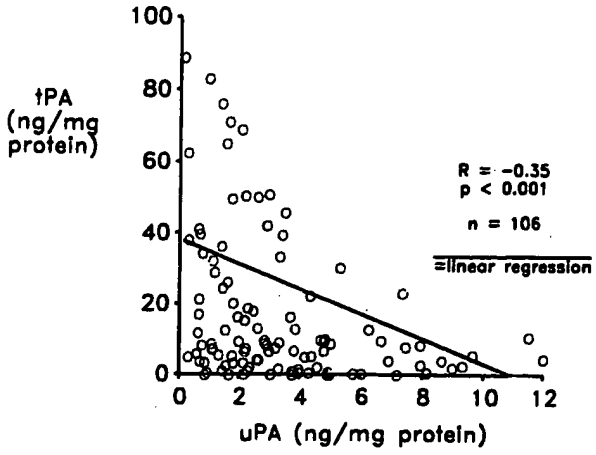

FIG. 7. Graphic representation of the correlation between u-PA and t-PA in breast cancer tissue extracts. A statistically significant inverse correlation was observed ($r = -0.35$; $p < 0.001$).

cells.[35] Still, this hypothesis needs further experimental evaluation.

Two different tumor-associated proteases, cathepsin B and D, have also been reported in regard to the metastatic potential of tumor cells in breast cancer. Cathepsin B, a cysteine-dependent protease, not only activates pro-u-PA,[8] but also displays proteolytic activity directed toward constituents of the tumor stroma.[36] There is no clinically related data on cathepsin B, so far. The aspartic protease cathepsin D, however, has been identified as a prognostic factor in breast cancer.[37,38] The direct evidence of cathepsin D's involvement in effective degradation of tumor stroma components remains to be elucidated. We did not see a correlation between cathepsin D (Table 1) and u-PA antigen in breast cancer tissues ($R = 0.12$, p = not significant). This seems to imply that synthesis and release of cathepsin D and u-PA are

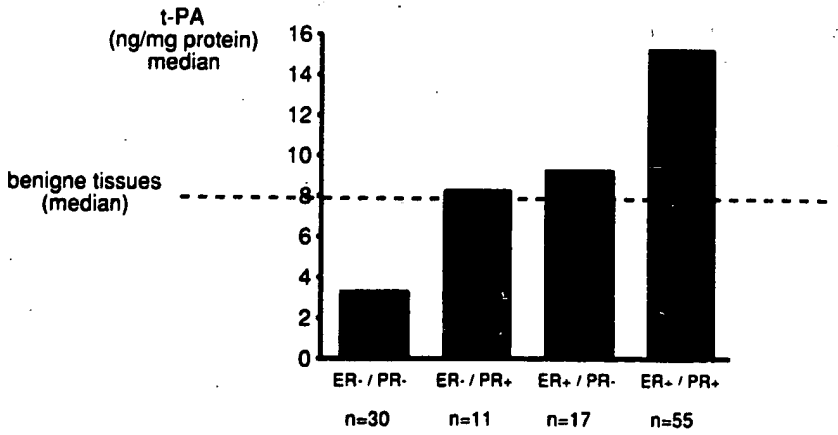

FIG. 8. Graphic representation of the correlation of t-PA and hormone receptor status in 113 breast cancer tissue extracts. ER: estrogen receptor; PR: progesterone receptor. The difference between t-PA content in ER(−)/PR(−) tumors (negative hormone receptor status) and each of the depicted other three subgroups of hormone receptor-positive tumors was statistically significant ($p < 0.01$ and $p < 0.001$, respectively).

regulated independently of each other. In any event, cathepsin D might be involved in the activation sequence of plasminogen to plasmin: Tumor cell-associated cathepsin D can activate procathepsin B to cathepsin B; cathepsin B then activates pro-u-PA to HMV-u-PA, which generates plasmin from plasminogen.[8]

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